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Trophectoderm Lineage Determination in Cattle

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SUMMARY

The trophectoderm (TE) and inner cell mass (ICM) are committed and marked by reciprocal expression of Cdx2 and Oct4 in mouse late blastocysts. We find that the TE is not committed at equivalent stages in cattle, and that bovine Cdx2 is required later, for TE maintenance, but does not repress *Oct4* expression. A *mouse Oct4* (*mOct4*) reporter, repressed in mouse TE, remained active in the cattle TE; *bovine Oct4* constructs were not repressed in the mouse TE. *mOct4* has acquired Tcfap2 binding sites mediating Cdx2-independent repression—cattle, humans, and rabbits do not contain these sites and maintain high *Oct4* levels in the TE. Our data suggest that the regulatory circuitry determining ICM/TE identity has been rewired in mice, to allow rapid TE differentiation and early blastocyst implantation. These findings thus emphasize ways in which mice may not be representative of the earliest stages of mammalian development and stem cell biology.

INTRODUCTION

A distinguishing feature of mammalian development is the early specification of the trophoblast/trophectoderm (TE), a subservient lineage contributing to the extraembryonic components of the placenta, essential for survival in the uterus yet unceremoniously discarded after birth. In the mouse the outer cells of the early (32 cell) blastocyst are already fated to form TE with inner cells destined to become the ICM (Dyce et al., 1987; Fleming, 1987). However, irreversible *commitment* to this fate occurs somewhat later in development. Thus, by the late (expanded) blastocyst stage (approximately 64 cell), ICM cells have lost the ability to form trophectoderm as seen by various assays (Balakier and Pedersen, 1982; Handyside, 1978; Rossant and Vijh, 1980). TE cells are committed slightly earlier than ICM cells (Spindle, 1978; Stern and Wilson, 1972) with a recent study showing that fate is fixed once cavitation commences in early blastocysts (Suwinska et al., 2008).

The restriction in fate of TE cells in the mouse involves the homeodomain-containing transcription factors Cdx2 and Oct4 (Pou5f1). At late blastocyst stages, these proteins display a reciprocal expression pattern with Cdx2 localized exclusively in the TE, and Oct4 in the ICM (Dietrich and Hiiragi, 2007; Strumpf et al., 2005). Both proteins are expressed ubiquitously from around the 8 cell stage. Four lines of evidence point to Cdx2 being required for TE lineage commitment. First, upon cavitation at the post 32 cell stage, a restriction of Cdx2 protein to outer cells (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008) occurs concomitantly with trophectoderm commitment. Second, upon functional inactivation of *Cdx2* (Strumpf et al., 2005), embryos form early blastocysts but fail to maintain their blastocoels as trophectoderm epithelial integrity is lost. TE cells apoptose and embryos fail to implant. Third, trophoblast stem (TS) cells, committed to the placental lineage, can be derived from normal (Tanaka et al., 1998), but not Cdx2 mutant embryos (Strumpf et al., 2005). Fourth, work in embryonic stem (ES) cells lends support to a maintenance role of Cdx2 for the TE lineage. ES cells represent the in vitro counterpart of committed ICM as they are able to contribute both in vivo and in vitro to all lineages apart from TE. Though repression of the pluripotency factor Oct4 can induce ES cells to differentiate into the trophoblast lineage even when *Cdx2* is deleted, self-renewing TS-like cells can only be derived if Cdx2 is expressed (Niwa et al., 2005). These four sets of experiments indicate a clear role for Cdx2 in TE commitment, but they also suggest that Cdx2 is not essential for the initial specification of the TE lineage. This is further supported by chimera studies in which Cdx2 deficient and wild-type two cell stage blastomeres were aggregated (Ralston and Rossant, 2008). Cdx2 mutant cells contributed to the TE with no bias toward the ICM.

How is Oct4 involved in TE commitment? Mouse Oct4 message and protein is normally downregulated in TE by the late blastocyst stage in line with it being essential for ICM but incompatible with TE development. In Cdx2 mutant embryos this TE-specific shutdown of Oct4 does not occur and TE development is impaired (Ralston and Rossant, 2008; Strumpf et al., 2005). Furthermore, in ES cells, either Oct4 downregulation or Cdx2 overexpression, which leads to Oct4 downregulation, causes differentiation along the trophoblast lineage (Niwa et al., 2005). This suggests that continued Oct4 expression prevents TE formation and that the downregulation of Oct4 is a primary function of Cdx2, required for stable TE maintenance. This downregulation appears to be direct as Cdx2 can interact with Oct4 at the distal autoregulatory *Oct4* enhancer to repress transcription (Niwa et al., 2005).

In contrast to the mouse, little is known in other mammals about early lineage commitment, though many studies have examined the expression of marker genes. Perhaps surprisingly, Oct4 protein does not appear to be restricted to the ICM of even expanded blastocysts in humans, cattle, pigs, or rabbits (Cauffman et al., 2005; Chen et al., 2009; Hall et al., 2009; Kirchhof et al., 2000; Kobolak et al., 2009; van Eijk et al., 1999), though one study did observe *Oct4* transcripts restricted to the ICM in

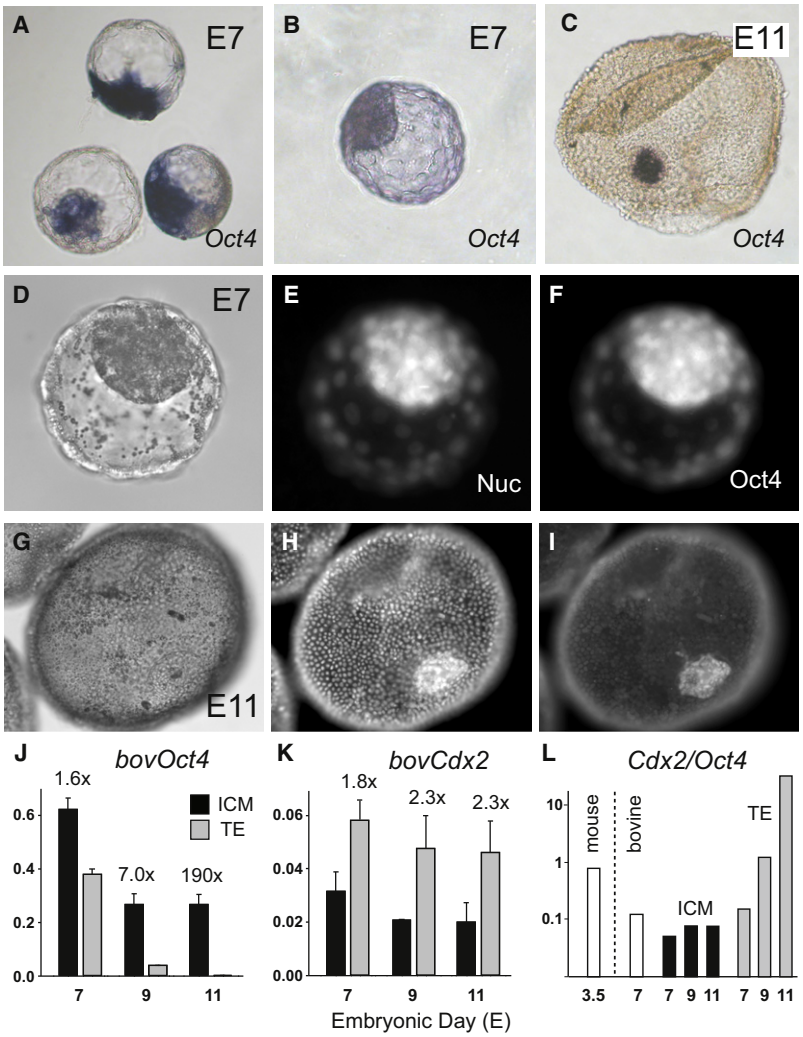

Figure 1. Localization and Quantitation of *Oct4* and *Cdx2*

(A and B) In expanded-blastocyst E7 cattle embryos, *bOct4* mRNA is seen predominantly (A), but not exclusively (B), in the ICM after whole-mount in situ hybridization. (C) Four days later, *bOct4* mRNA is restricted to the epiblast. (D–F) bOct4 protein expression is ubiquitous at E7 (F), as revealed by comparison to Hoechst nuclear staining (E). (G–I) By E11, bOct4 protein is seen only in the epiblast. (J and K) Bovine *Oct4* (J) and *Cdx2* (K) expression normalized against the geometric mean of *Gapdh, Cyclophilin* and *HPRT*. Numbers above bars show the fold enrichment in the respective tissue, error bars in all figures are SEM. (L) At blastocyst stages, the whole blastocyst *Cdx2:Oct4* ratio (empty bars) in mice is nearly 10-fold higher than in cattle where an equivalent ratio is only reached specifically in the TE at E9.

cattle (Kurosaka et al., 2004). At later developmental stages, *Oct4* RNA and protein appears to be restricted to the epiblast (Degrelle et al., 2005; Hall et al., 2009; Vejlsted et al., 2005), though there is, in pigs, also evidence to the contrary (Keefer et al., 2007). Regarding trophoblast markers, human and cattle blastocysts transcribe *Cdx2* though transcripts were not spatially defined nor quantified (Degrelle et al., 2005; Kimber et al., 2008). Cdx2 protein appeared to be restricted to the TE in pig and cattle blastocysts (Kuijk et al., 2008). Speculations abound in the literature as to the meaning of potential differences in marker gene expression between mice and other mammals.

Cattle embryos take 4 days from fertilization to reach the 16 cell stage when inner and outer cells can first be distinguished. In vivo, blastulation commences at embryonic day 6 (E6) when the embryo consists of 100 cells, expanded (late) blastocysts are seen from E7 and hatching commences at E8 (Van Soom et al., 1997). Cattle embryos are routinely grown in vitro for 7 days post-fertilization (E7) and reach morphological landmarks up to a day before their in vivo counterparts (Van Soom et al., 1996). By day 8, the ICM has begun differentiating into epiblast and hypoblast (primitive endoderm) and 2 days later the hypoblast layer has completed lining the inner surface of the TE (Maddox-Hyttel et al., 2003). The epiblast is termed the embryonic disc once the overlying trophectoderm (Rauber's layer) disappears, at around E12. Gastrulation and mesoderm formation commence at E14.

Using cattle as our nonrodent model, we set out first, to quantify expression levels of the factors that have been shown to be important for lineage commitment in the mouse, second, to determine why differences exist, third, to functionally analyze the role of said factors and finally, to examine whether the trophoblast of the expanded bovine blastocyst is committed or not.

RESULTS

Oct4 Expression in Cattle Preimplantation Embryos

In light of contradictory reports on Oct4 distribution in different mammals, we measured bovine Oct4 expression in a qualitative as well as quantitative manner. We noted variable staining, when performing whole-mount in situ hybridization (WMISH) on late-stage (expanded E7) cattle blastocysts. Whereas the ICM always contained *Oct4* RNA, TE exhibited no (Figure 1A) or significant (Figure 1B) staining, depending on the experiment. In contrast, by E11, only epiblast cells exhibit *Oct4* expression (Figure 1C). As WMISH is not very quantitative, we isolated ICM/epiblast and TE tissue and measured expression by real-time RT-PCR (Figure 1J). TE of E7 cattle blastocysts contained quite high amounts of *Oct4* RNA, amounting to 60% of those seen in the ICM. By E9, TE levels were 14% of ICM levels. Minimal expression was detectable in E11 TE. Concomitantly, overall amounts of *Oct4* in the ICM/epiblast dropped significantly between E7 and E9 (Figure 1J), presumably because the ICM has differentiated into expressing epiblast and nonexpressing hypoblast/primitive endoderm during this time (Maddox-Hyttel et al., 2003). The quantified distribution of *Oct4* message is reflected at the protein level where we observed measurable amounts of Oct4 protein in the TE of E7 blastocysts but none by E11 (Figures 1D–1I).

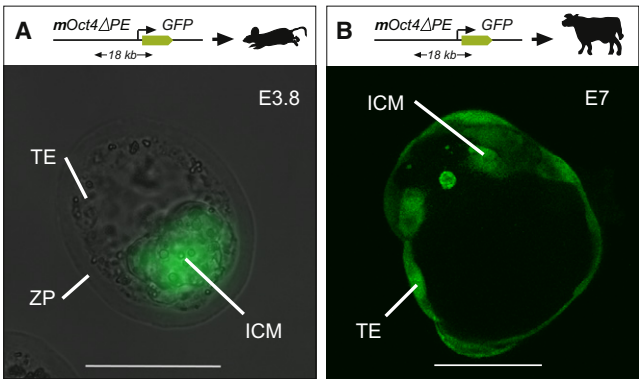

Figure 2. Expression of a Mouse Oct4 Reporter Is Not Downregulated at the Blastocyst Stage in Cattle TE

(A) mOct4ΔPE-GFP expression in mouse late blastocysts.

(B) mOct4ΔPE-GFP expression in cattle late blastocysts. Bar: (A), 50 μm; (B), 100 μm; ZP, zona pellucida.

Trans-Regulative Differences in Mammalian *Oct4* Expression

Why is Oct4 expression maintained in cattle, but not mouse, late blastocyst trophectoderm? Potentially, the bovine preimplantation embryo does not contain the regulative factors necessary for shutdown of *Oct4* transcription. We pursued this possibility using the mouse Oct4 reporter mOct4ΔPE-GFP, which faithfully recapitulates endogenous preimplantation *Oct4* expression (Yoshimizu et al., 1999). Thus, in mice, by late blastocyst stages GFP expression is confined to the ICM (Figure 2A). We next used this construct to stably transfect bovine primary fibroblast cells. These cells were then used for somatic cell nuclear transfer (SCNT) to produce transgenic cattle embryos. SCNT does not interfere with normal *Oct4* expression in cattle (Smith et al., 2007). Similar to the endogenous *Oct4* expression pattern, mOct4ΔPE-GFP embryos fluoresced ubiquitously at the 8 cell stage, concomitant with the major burst of embryonic gene activation (see Figure S1 available online). Notably, reporter expression was maintained in the TE, even in very late-stage expanded blastocysts (18/18 embryos) (Figure 2B; Figure S1). This experiment shows that at late blastocyst stages, cattle TE cannot downregulate the *Oct4* gene construct even in the presence of *cis*-regulatory elements sufficient for shutdown in the mouse. Thus, cattle TE must contain a different mix of *trans*-acting regulatory factors.

Cdx2 in Early Cattle Preimplantation Development

In the mouse, Cdx2 has been implicated in both TE lineage commitment and Oct4 downregulation in the TE. In view of the lack of Oct4 as well as mOct4ΔPE-GFP downregulation in TE of cattle blastocysts, we examined *Cdx2* expression in this species. *Cdx2* mRNA could be detected between E7 and E14, dropping to background levels by E17 (Figure 1K; data not shown). Expression was enriched in the TE relative to the ICM by 2-fold (Figure 1K). Notably, levels of *Cdx2* in the TE were very low, being 6.6-fold lower than *Oct4* in E7 late blastocysts (Figure 1L). In contrast, *whole* mouse blastocysts contained about equal levels of *Oct4* and *Cdx2* RNA. Considering the exclusive distribution pattern of these genes in the mouse, *Cdx2* levels far exceed those of *Oct4* in the TE (Figure 1L). Mammals contain two paralogs of Cdx2, namely, Cdx1 and Cdx3/4. Neither paralogue was expressed at blastocyst stages in cattle though we did detect, in oocytes, maternal *Cdx1* which was degraded by the 8 cell stage (data not shown). Thus, the low levels of *Cdx2* in the bovine embryo are not compensated for by expression of paralogous genes.

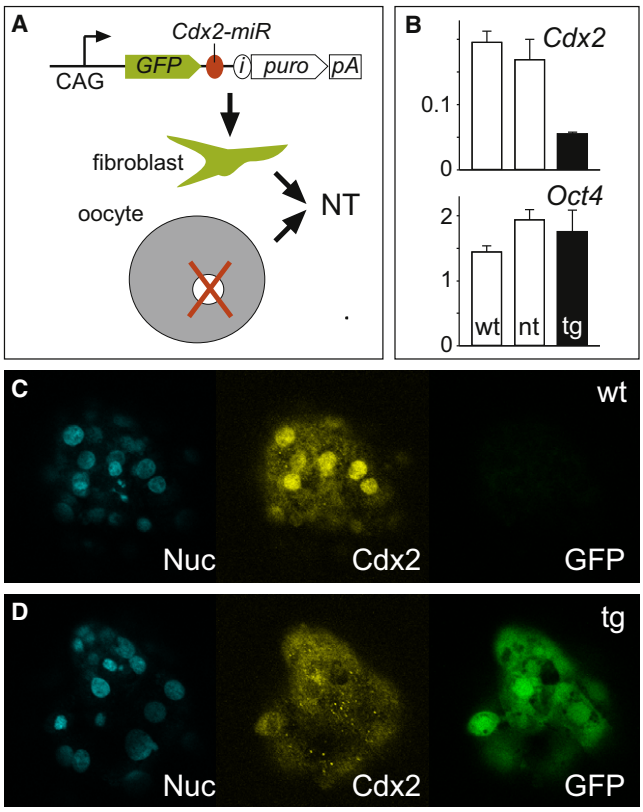

Figure 3. *Cdx2* Knockdown in Cattle Blastocysts

(A) Strategy to generate cattle Cdx2 knockdown embryos.

(B) *Cdx2* mRNA in E7 blastocysts dropped to near-background levels which did not affect endogenous *Oct4* expression (real-time PCR). Wt, wild-type; nt, nuclear transfer controls; tg, transgenic embryos.

(C) Confocal image of the TE of a late blastocyst embryo stained for Cdx2 protein. Cdx2 staining (middle) colocalizes with nuclei (left) visualized with Bisbenzimide 33342.

(D) Cdx2 knockdown blastocyst loses nuclear Cdx2 expression while exhibiting Emerald-GFP fluorescence (right).

Considering the low and constant levels of bovine *Cdx2* between E7 and E11 the question arises as to whether Cdx2 is actively involved in *Oct4* downregulation, as has been shown for the mouse. We constructed two *pCAG*-driven, miR-based siRNA vectors to knock down *Cdx2* mRNA and tested these in vitro by cotransfection with a target plasmid containing the siCdx2 target sequence inserted behind a *pCMV*-driven DsRed reporter. Bovine fibroblasts were stably transfected with the knockdown constructs and, after in vitro validation, used for transgenic embryo generation (Figure 3A). One of the siRNA constructs, *Cdx2-miR712*, knocked down *Cdx2* RNA in vivo by 72%, resulting in an absence of detectable nuclear Cdx2 protein (Figures 3B–3D).

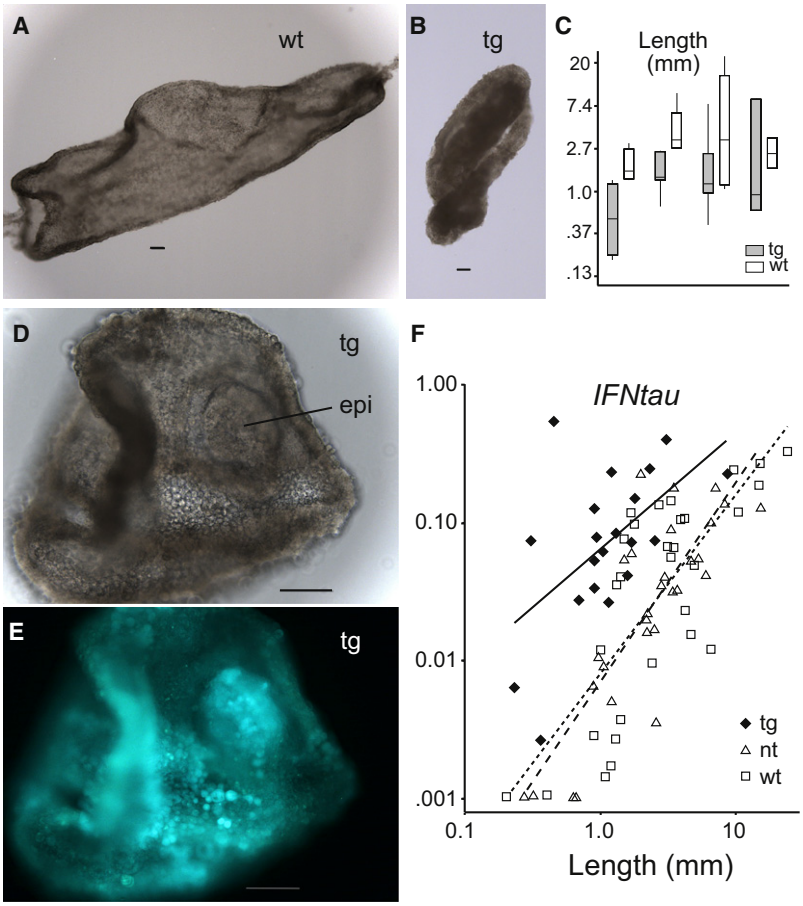

Figure 4. *Cdx2* Knockdown Affects TE but Not Epiblast Development

(A and B) Representative examples of wild-type (A) and transgenic (B) cattle E14 embryos demonstrate size retardation.

(C) The size effect is logarithmically graphed in box (lower and upper quartiles) and whisker (data range) plots for four sets of nuclear transfer experiments with wt and tg embryos for each experiment shown side by side.

(D and E) Embryo (E14) showing well developed, normal-sized epiblast but severe reduction of TE tissue and continued expression of miR-based *siCdx2* via co-cistronic GFP fluorescence.

(F) Relation of mRNA levels of *Interferon-tau* to embryo length in E14 and E15 embryos. Regression lines are solid for tg, dashed for nuclear transfer control and dotted for wild-type control embryos. Scale bars, 100 μm.

Development to blastocyst and blastocoel and TE formation in *Cdx2* knockdown embryos were as expected for nuclear transfer derived embryos (Table S1). Notably, at E7, *Oct4* RNA levels were normal, indicating that in the bovine blastocyst, Cdx2 does not significantly repress *Oct4* transcription (Figure 3B).

The Role of Cdx2 in Cattle TE after Blastocyst Stages

The low levels of Cdx2 in cattle TE and its dispensability for blastocyst formation and *Oct4* transcriptional repression raises the question whether this gene is required at all for TE development in this species. In the mouse, the mesoderm marker Eomes is also involved in TE formation where it is regulated by Cdx2. We were unable to detect, by RT-PCR, Eomes in TE between E7 and E15, although we could easily measure its expression from E14 in epiblast tissue of cattle gastrula-stage embryos (data not shown). This indicates that Cdx2's role (if any) in the bovine TE is independent of Eomes. We followed the development of Cdx2 knockdown embryos to the beginning of gastrulation, by transferring blastocysts to the uteri of synchronized recipient cows and nonsurgically flushing the tracts a week later. As the recipient can have a large effect on developmental rate (Berg et al., 2010), transgenic and control embryos were transferred into the same host. Cdx2-knockdown embryos were significantly shorter than controls (Figure 4). The reduction in length can be attributed to a lack of TE proliferation, as it is the TE that makes up the bulk of the embryo. Interestingly, embryos were retrieved with a well-formed epiblast (embryonic disc) characteristic of an E14 embryo and expressing *Oct4* (in the epiblast) at levels similar to size matched controls (Figure S2), yet contained abnormally few TE cells (Figures 4D and 4E). In cattle, expression of the trophoblast-specific pregnancy recognition factor Interferon-τ (Ifn-τ) increases exponentially around E14. To analyze the developmental state of the TE in Cdx2-knockdown embryos, we compared *Ifn-τ* expression to size matched nuclear transfer and wild-type embryos. Wild-type and nuclear-transfer generated embryos expressed *Ifn-τ* in proportion to their length (Figure 4F). In marked contrast, small knockdown (epiblast containing) embryos expressed *Ifn-τ* at levels characteristic of much larger embryos (Figure 4F), indicating that whereas TE proliferation is affected, the developmental program of the TE cells is not retarded.

Cis-Regulative Differences in Mammalian *Oct4* Expression

Is the species-specific difference in *Oct4* transcription in the TE caused solely by (developmentally time-dependent) differences in regulatory factors such as Cdx2? If so, we would expect that the bovine *Oct4* locus would be shutdown in mouse TE. To test this, we used recombineering technology to construct *bovOct4-GFP*, a bovine reporter equivalent to mOct4ΔPE-GFP, containing 18 kbp of bovine *Oct4* regulatory regions, but without the proximal, epiblast-specific enhancer deletion. Nuclear transfer derived cattle blastocysts transgenic for *bovOct4-GFP* exhibited fluorescence in both ICM and TE (32/32 embryos) (Figure 5A), in a pattern analogous to the endogenous Oct4 protein (Figure 1). We next microinjected *bovOct4-GFP* into mouse zygote pronuclei and retrieved these from recipient mice at the late blastocyst stage. Interestingly, GFP expression remained strong in the TE (5/5 transgenic embryos) (Figure 5B). This shows that the mouse TE, though containing the factors necessary to switch off the mouse *Oct4* locus, is unable to extinguish transcription from the bovine locus. We conclude that both the regulatory factor repertoire/activity in

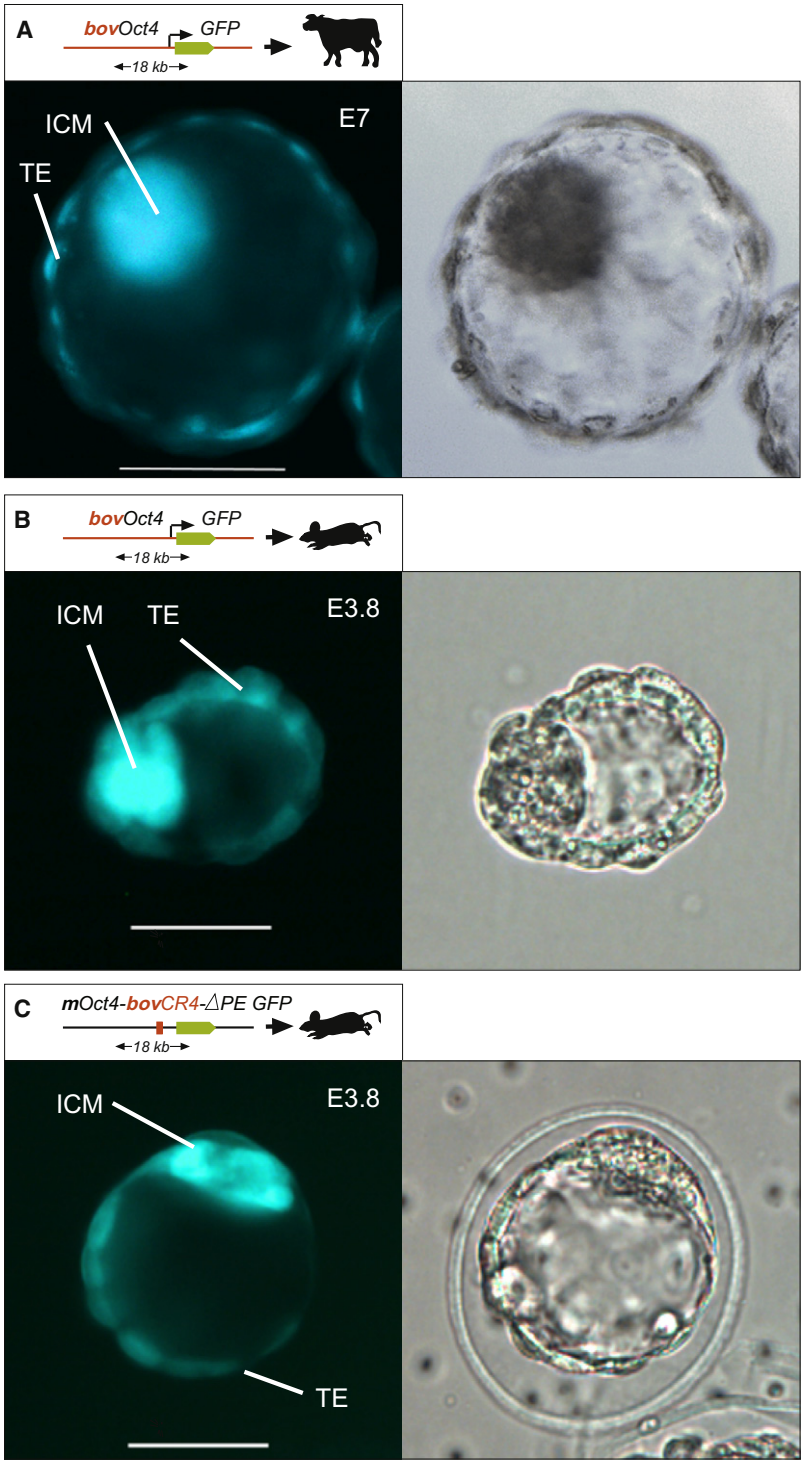

Figure 5. Blastocyst Species-Specific Differences in *cis*-Regulation of Mouse and Cattle *Oct4* Reside within the CR4 Region

(A) bovOct4-GFP expression in late-stage cattle blastocysts.

(B) bovOct4-GFP expression in mouse embryos (hatched blastocyst shown).

(C) Ubiquitous expression of the mouse mOct4ΔPE-GFP with the mouse CR4 replaced by the bovine CR4. Bar: (A), 100 μm; (B and C), 50 μm.

the blastocyst TE, as well as the *cis*-regulatory region of *Oct4* have diverged between these species.

The Role of CR4 within the ICM-Specific Distal Enhancer of Oct4

A comparison of the mouse, bovine, and human *Oct4* loci has revealed four upstream conserved regions termed CR1 to 4 (Nordhoff et al., 2001). The upstream-most CR3 and CR4 regions form part of the distal enhancer, required for expression in the morula, inner cell mass as well as for primordial germ cell expression (Yeom et al., 1996; Yoshimizu et al., 1999). CR4 contains the autoregulatory Oct4/Sox2 element (Chew et al., 2005). Sequence comparisons reveal the conservation of the autoregulatory element but only 66% identity overall in the CR4 between mouse and bovine/human as opposed to 85% between human and bovine, whereas CR1 to 3 show between 77% and 94% identity across all three species (Nordhoff et al., 2001). The higher sequence divergence in CR4 prompted us to focus on this region. We replaced the mouse CR4 with that of cattle in the mouse mOct4ΔPE$_{18}$-GFP reporter and generated transgenic mouse blastocysts. Remarkably, this construct behaved like the bovine Oct4-reporter construct, with robust GFP expression seen in the TE of late blastocysts (4/4 transgenic embryos) (Figure 5C). We conclude that the 246 bp mouse CR4 region is necessary for extinguishing *Oct4* expression in the TE.

Differences in Protein Binding between Mouse and Cattle CR4

Considering that the mouse but not bovine CR4 region can exert an inhibitory influence on Oct4 expression, we next asked whether these regions bound different factors. Mouse ES cell extracts, representative of the ICM, yielded three major bands upon electophoretic mobility shift assays (EMSA) with both mouse and bovine CR4 probes (Figure 6B, lanes 2 and 7). Using competitive oligos (lanes 3–5, 8–10), we could show these bands to correspond to binding to the highly conserved Oct/Sox and the two Zn-finger protein binding sites (Figure 6A). We then performed EMSA using mouse TS cell extracts representative of polar TE. Notably, such extracts bound to the mouse, but not bovine CR4 probe (Figure 6B, lane 1 versus 6). The factor responsible for this binding activity was not exclusive to mouse TS cells. Extracts prepared from differentiated mouse trophoblast (TS cells incubated for 5 days without growth factors) or from a feeder-free bovine TE cell line yielded the same bands (Figure 6B, lanes 11 and 33).

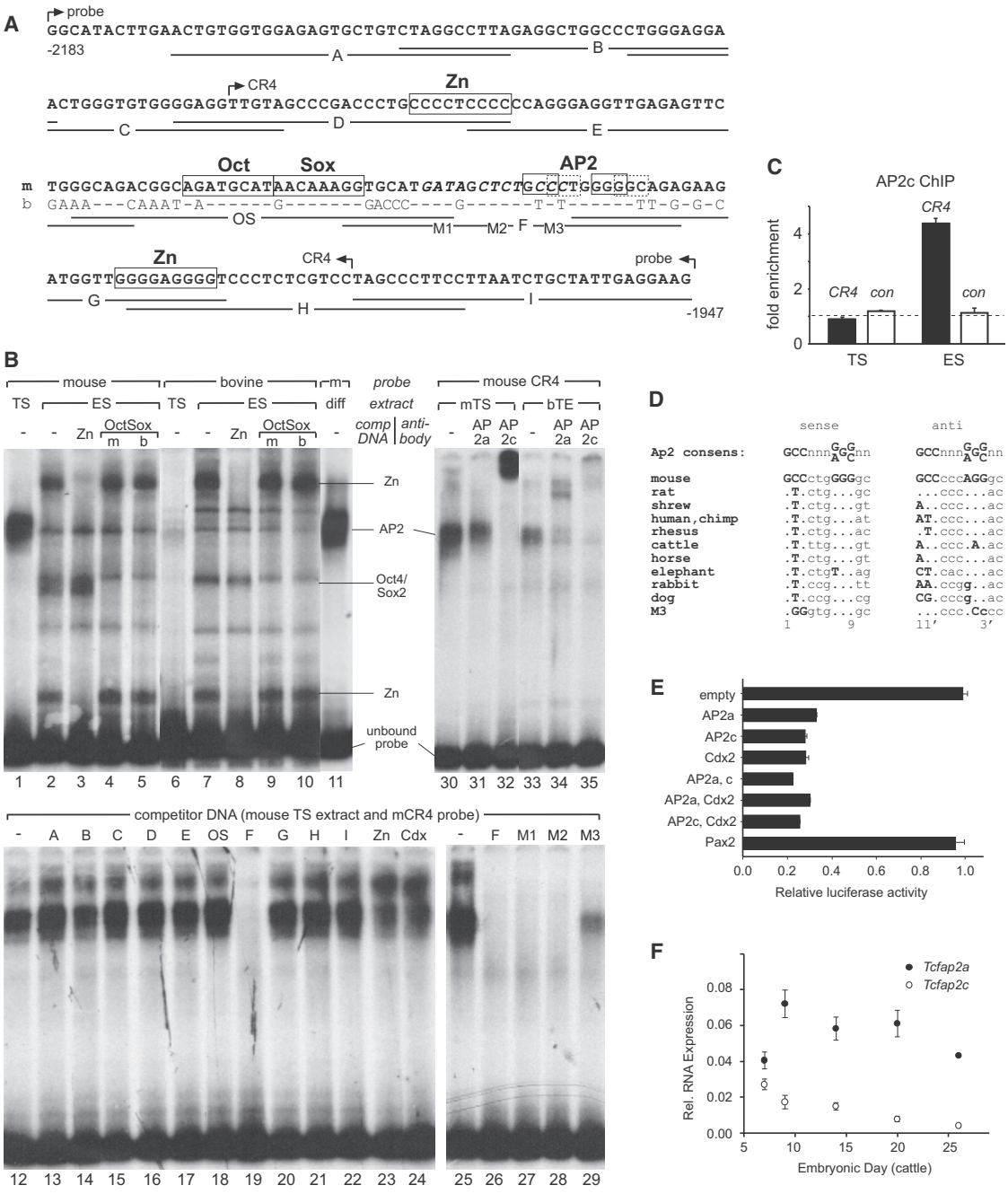

Figure 6. Analysis of the CR4 Region of *Oct4*

(A) Mouse CR4 sequence used as probe for EMSA. Zinc-finger protein (Zn), Oct4/Sox2 (Okumura-Nakanishi et al., 2005), and Tcfap2 (AP2) binding sites are boxed. Note the overlapping AP2 sites (GCCnnnGGG) in opposing orientation. Cattle sequences (b) are shown where relevant, with dashes indicating nucleotides identical to mouse. Sequences used for competition oligos are underlined. Regions mutated in oligo F to delineate binding sites (M1:*ACAT*; M2:*AACC*; M3:*GGG*) are italicized. Numbering relative to translational start site.

(B) EMSA using radioactively labeled mouse *Oct4 CR4* probe, or for lanes 6–10 bovine CR4, with extracts, competitive oligos and antibodies as indicated.

(C) AP2γ ChIP of mouse TS and ES cells followed by quantitative PCR for *Oct4* CR4 and a control *Elf5* region. Enrichment relative to nonimmune serum is shown.

(D) Sequence comparison of the two overlapping AP2 sites among several mammals. The second site is located on the antisense strand and thus shown reverse complemented.

(E) Luciferase activity relative to cotransfected TK-RL of mouse CR4-Prom-luciferase in ES cells. Equal amounts of empty pKW2T vector or full length mouse AP2α, AP2γ, Cdx2, and Pax2 were cotransfected.

(F) Expression of *Tcfap2a* and *Tcfap2c* relative to the geomean of three housekeepers in E7, E9, and E14 whole cattle embryos and E20, E26 TE.

Three lines of evidence indicated that this binding activity was not Cdx2. First, in vitro translated Cdx2 could not bind to mCR4 (data not shown). Second, binding was not affected by competition with an optimal Cdx2 binding site (Figure 6B, lane 24). Third, anti-Cdx2 antibodies neither super-shifted nor removed the bands (data not shown).

We identified the region responsible for the mouse-CR4 specific binding to the 30 bp lying between the Oct/Sox and the second Zn-finger binding sites by using competitive oligos covering the entire mCR4 region (Figure 6B, lanes 12–22). Sequence inspection followed by competition EMSA with site-specific mutations in these 30 bp suggested that binding was due to an AP2 (*Tcfap2* gene family) factor (Figure 6B, lanes 26–29). Indeed, neither of the two overlapping predicted AP2 high affinity sites (lying on opposite strands, see Figures 6A and 6D) in mCR4 were present in the bovine CR4 (Figure 6A). Two members of the AP2 protein family are expressed in the preimplantation trophectoderm: AP2α and AP2γ (Winger et al., 2006), with the gamma isoform being predominant in TS cells (Kuckenberg et al., 2010). These proteins do not differ in their binding site specificity (McPherson and Weigel, 1999) so we tested for both proteins. Antibody supershifts indeed identified the AP2γ protein family member to be responsible for all of the TS cell mCR4-binding activity (Figure 6B, lanes 31 and 32), while in the bovine trophectodermal cell line, AP2α also contributed to binding (lanes 34 and 35).

Whereas AP2γ bound strongly to naked DNA, no significant binding to the Oct4 CR4 region could be detected in TS cells via ChIP assays (Figure 6C; Kidder and Palmer, 2010), potentially due to the heterochromatic state of the Oct4 gene in these cells. To simulate a more open chromatin configuration as would be expected in blastomeres, we repeated the ChIP assay in ES cells. Low though significant binding of AP2γ could be detected using PCR primers covering CR4 (Figure 6C). That binding would be weak was expected as ES cells do not express much of this protein (Figure 6B, lanes 2–5).

We conclude that the mouse and cattle ICM enhancers of *Oct4* differ in their ability to bind to AP2 proteins. Only the mouse CR4 region is able to bind to AP2α or γ. Second, binding is dependent on the chromatin state of the Oct4 locus.

Effect of AP2 Proteins on *Oct4* Transcription

Are AP2 proteins involved in the selective shutdown of mouse but not cattle Oct4 expression in the late blastocyst TE? If so, it might be possible to mimic this repressive activity in ES cells. ES cells transfected with a mouse CR4-Oct4Prom-Luciferase reporter and an empty expression vector showed robust expression relative to a control TK-renilla luciferase reporter. Cotransfection with an expression vector driving *Cdx2* severely reduced expression of the reporter (Figure 6E), as previously shown by others (Okumura-Nakanishi et al., 2005). Notably, a similar effect was seen when cotransfecting with either *Tcfap2a* or *Tcfap2c* (Figure 6E). Combinations of factors did not further reduce the relative luciferase activity of the *Oct4-CR4* enhancer. We conclude that AP2α and AP2γ act as transcriptional repressors of *Oct4*, independently of Cdx2. This finding is particularly pertinent in view of the fact that AP2γ has recently been implicated in the induction of trophoblast fate (Kuckenberg et al., 2010) and AP2α is specifically upregulated in late blastocyst TE when Oct4 is downregulated (Guo et al., 2010).

We measured the abundance of *Tcfap2a* and *Tcfap2c* in cattle during development (Figure 6F). Transcript levels for these two genes were similar in E7 blastocysts with Tcfap2a being somewhat more abundant. Thereafter, *Tcfap2a* increased then steadied out in TE while *Tcfap2c* slowly decreased over a large developmental time span. Both AP2 proteins were also present in the bovine TE cells (Figure 6B, lanes 34 and 35). The presence of these factors in the bovine blastocyst TE when Oct4 levels are high re-enforces the idea that in this species AP2 proteins are not involved in lowering Oct4 transcription.

Lineage Fate and Commitment of Cattle TE

Is there a biological significance to the difference in Oct4 regulation? Potentially, the slow decay of Oct4 expression in the cattle TE could delay lineage commitment of this tissue. Before addressing commitment, we followed the fate of E7 TE cells during the following 7 days, in which the hypoblast forms, the polar trophectoderm (named Rauber's Layer) disappears and gastrulation commences. The entire TE layer was labeled using the lipophilic dye, CM-DiI (Figure 7A), and blastocysts transferred into synchronized recipient cows. Embryos were retrieved 1 week later. In all cases, labeled TE cells did not contribute to the epiblast (Figures 7B and 7C), indicating that TE cells are indeed fated to remain trophectoderm when not perturbed.

We next addressed TE commitment by mixing labeled, dissociated E7 TE cells with E3 8 cell blastomeres and allowing these chimeras to develop to the blastocyst stage (Figure 7D). Notably, approximately one-third of the TE cells contributed to the ICM (Figure 7E; Table S2). To determine whether older TE is committed as judged by this assay, we repeated the experiment using labeled, dissociated TE cells from E14 gastrulation-stage embryos (Figure 7F). Such E14 TE cells (expressing very little *Oct4*) contributed nearly exclusively to the TE of resulting chimeric blastocysts (Figure 7G; Table S2).

To further exclude the possibility that the E7 TE cells had simply been trapped on the inside of the embryo, we followed the fate to later stages. Owing to progressive dilution of the dye and the relatively slow development of the bovine embryo, we could not use DiI labeling. Instead, we marked host cells genetically by generating nuclear transfer-derived embryos using bovine fibroblasts expressing β-galactosidase constitutively from a CAG enhancer. Wild-type E7 TE cells from IVP embryos were aggregated to such β-Gal 8 cell NT embryos. The chimeras were transferred to recipients and 3 embryos retrieved at E16, after the onset of gastrulation. Two of the embryos were chimeric as evidenced by patches of white cells after staining for β-Gal. Notably, in one embryo (Figure 7I), white hypoblast cells were seen, indicative of contribution of the donor E7 TE cells to this ICM-derived tissue.

We conclude that the high level of Oct4 in the blastocyst TE of cattle correlates with a lack of commitment of this tissue.

DISCUSSION

In mice, the *specification* of the TE lineage from the pluripotent early blastomeres involves the Hippo signaling pathway with activation of the Tead4 transcription factor at morula stages

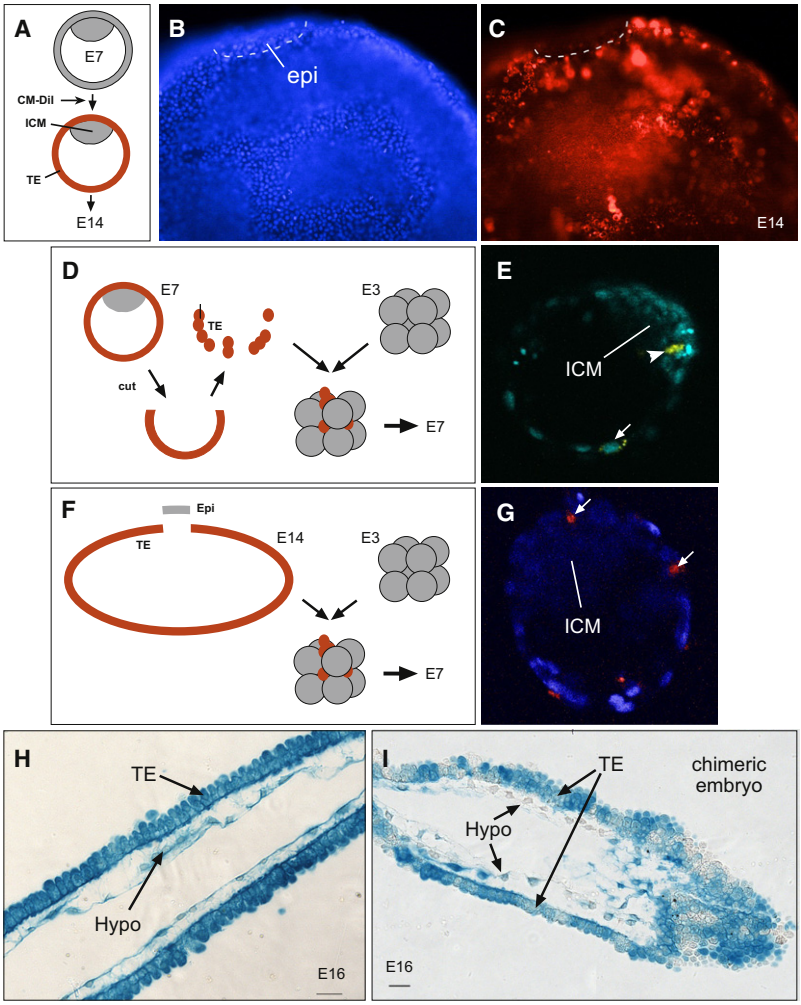

Figure 7. E7 Cattle Trophectoderm Cells Are Fated to Remain TE but Are Not Committed to This Fate

(A) Strategy for following TE fate.

(B and C) None of the epiblast cells, revealed in (B) by Hoechst staining, were labeled by DiI (C).

(D and E). Trophectoderm cells are not committed at blastocyst stages. (D) Strategy for assessing TE commitment. (E) Confocal image depicting a labeled cell in the TE (arrow) and ICM (arrowhead).

(F and G) Trophectoderm cells of pregastrulation stage embryos are committed. (G) Confocal image of E7 blastocyst showing typical nearly exclusive contribution of labeled E14-TE derived cells to the TE.

(H and I) Long-term development of aggregation chimeras with nuclear-transfer derived host embryos positive for LacZ and wt E7-TE reveal E7 TE contribution to the ICM-derived hypoblast in E16 chimeric embryo cross-sections (I). Nonsuccessful aggregations (controls) resulted in all hypoblast cells staining blue (H). Scale bar, 50 μm.

playing a decisive role (Nishioka et al., 2009; Yagi et al., 2007). This mechanism may also be conserved in cattle as we were able to detect bovine *Tead4* RNA in cattle morula (data not shown). Thus, by early blastocyst stages cattle and mouse embryos are characterized by two morphologically distinct cell populations of reciprocal fates: the ICM and outer trophectoderm. This morphological difference is of functional significance in the mouse as these lineages have been shown to be *committed* to their fate by the mid blastocyst stage. In marked contrast, while TE cells of the late expanded cattle blastocyst are fated to remain trophectoderm, they are not yet committed to this fate, as shown in our aggregation assays.

Commitment requires the establishment of a self-enforcing transcriptional network that is characteristic of the lineage and prevents the cell from following an alternate lineage. The transcription factors Cdx2, Eomes, Tcfap2c, Elf5, Ets2, Sox2, and Gata3 are key early players in maintaining the trophectoderm lineage in mice as loss of function studies result in (1) the loss of proliferating TE cells and (2) the inability to derive TS cells (Avilion et al., 2003; Donnison et al., 2005; Georgiades and Rossant, 2006; Home et al., 2009; Kuckenberg et al., 2010; Ralston et al., 2010; Strumpf et al., 2005). We have here shown that Cdx2 is also important for maintaining the trophectoderm lineage in cattle. However, a network simply maintaining trophectoderm characteristics does not suffice for commitment, as trophectoderm morphology precedes commitment in both mice and cattle. The trophectoderm network also has to prevent development along the alternate fate of the early blastomere, that of the pluripotent ICM state. At the core of the pluripotent network lie the genes Oct4, Nanog, and Sox2 (Boyer et al., 2005). These genes not only autocatalytically maintain each other's expression but also repress a host of genes involved in other lineages including that of the trophectoderm. For example, Oct4 has been shown to downregulate *Cdx2* expression in ES cells and Oct4 binding sites have been detected in the upstream promoter and intron 1 region of *Cdx2* (Loh et al., 2006; Niwa et al., 2005). The pluripotent network is clearly very dominant in that artificial temporary overexpression of various subsets of the key genes, but always including Oct4, can induce even differentiated cells to revert to an ES-like (iPS) state (Takahashi and Yamanaka, 2006). Conversely, Oct4 repression in ES cells leads to a trophectoderm fate (Niwa et al., 2005). The interconversion between these lineages may not be so surprising as the ICM and TE are ontogenetically closely related, sharing a common Oct4-expressing predecessor (the morula stage blastomere). It follows that shutdown of Oct4 activity and thereby of the pluripotency network is a primary requirement for the trophectoderm network to achieve autonomy. Interestingly, when and how this shutdown of Oct4 is achieved, varies among mammals, as shown here.

Of the key trophectoderm transcription factors, Cdx2 has been singled out as critical as it is the only factor shown to directly interfere with Oct4 activity. Cdx2 can interfere with Oct4 autoregulation within the conserved region 4 (CR4, also termed ARE) of the distal *Oct4* enhancer, resulting in transcriptional repression (Niwa et al., 2005). This inhibitory effect may be more widespread as these authors showed Cdx2 to bind to

Oct4, thus potentially also directly affecting the transcription of hundreds of Oct4 target genes. Notably, in cattle late blastocysts, Oct4 expression remained strong in the TE even though Cdx2 mRNA and protein were enriched in this tissue. Furthermore, reducing endogenous *Cdx2* levels in the bovine TE did not increase *Oct4* expression, suggesting that bovine *Oct4* expression is not significantly repressed by Cdx2 at the late blastocyst stage in cattle. The likely reason for this lack of repression is the striking difference in *Cdx2* to *Oct4* ratios between mouse and cattle blastocysts. Whole cattle late blastocysts display a nearly 10-fold lower *Cdx2:Oct4* mRNA ratio than mouse late blastocysts with the difference even more pronounced when comparing TE tissue only. In cattle TE, *Cdx2* transcript levels only start exceeding *Oct4* levels after E9 (at epiblast stages when the hypoblast has nearly completed circumnavigating the blastocoel cavity), whereas the 64 cell mouse late blastocyst already exhibits near-exclusive expression of these two factors in the TE and ICM respectively. Why is the Cdx2:Oct4 ratio important? According to Niwa's report, the Cdx2:Oct4 complex inhibits the activity of both proteins (Niwa et al., 2005). Thus, an excess of Oct4, as in cattle, would mop up Cdx2 activity thereby preventing the shutdown of *Oct4* expression. This is exactly what we saw when introducing the mouse *Oct4-GFP* construct into cattle: though the construct contained the regulatory regions necessary for TE inhibition in the mouse, it remained active in the cattle TE environment.

If the Cdx2:Oct4 ratio in trophoblast cells were the only distinguishing feature between cattle and mice, we would have expected the bovine Oct4-GFP reporter to be switched off in mouse TE. This was not the case and showed that the bovine Oct4 locus does not contain the *cis*-acting regulatory region necessary for extinguishing transcription in blastocyst TE. We could pin down the critical region to the CR4 sequence in the distal enhancer by swapping the mouse CR4 for the bovine CR4 in the context of 18 kbp of the proximal-enhancer-less Oct4 locus (the proximal enhancer has been shown to direct epiblast and germ cell expression of Oct4; (Yeom et al., 1996)). This led to the discovery of two overlapping high affinity AP2 (Tcfap2) binding sites located between the highly conserved autoregulatory Oct4/Sox2 and the second Zn-finger-protein binding sites. These AP2 sites were not present in cattle. Intriguingly, sequence inspection revealed that these sites were unique to mice with the closely related rat presenting the intermediate situation of only one predicted functional site (Figure 6Z). Interestingly, humans, rabbits, and pigs, similar to cattle, do not downregulate Oct4 in the TE at blastocyst stages (Cauffman et al., 2005; Kobolak et al., 2009; Kuijk et al., 2008). Our luciferase data indicates that both AP2α and γ can indeed repress the CR4-driven expression of Oct4 in ES cells.

The Oct4-repressive role of the AP2 proteins fits well with recent data demonstrating an instructive role for Tcfap2c in the generation of TS cells from ES cells (Kuckenberg et al., 2010). The timing of expression of the *Tcfap2* genes is compatible with an involvement in TE commitment. At the blastocyst stage *Tcfap2a*, *c*, and *e* (AP2α, γ, and ε) are expressed, though mRNA levels of *Tcfap2e* were too low to be detectable by in situ hybridization (Winger et al., 2006). Notably *Tcfap2a* commences only at the 16 cell morula stage with expression clearly restricted to the TE by the 32 cell blastocyst stage (Guo et al., 2010; Winger et al., 2006). The more ubiquitous AP2γ protein is high in the TE and reduced in the ICM by the late blastocyst stage (Kuckenberg et al., 2010). As the double loss of function of *Tcfap2a* and *2c* is more severe in terms of embryo survival than loss of either gene, genetic redundancy exists (Winger et al., 2006). Thus, both factors are likely to contribute to *Oct4* repression as well as TE-network activation, potentially in a dose-dependent fashion.

We surmise that mice use at least two distinct mechanisms to actively repress Oct4 function in the TE. One involves Cdx2, the other AP2 proteins. While both factors affect Oct4 transcription, Cdx2 is likely to be more effective in that it will also affect the activity of the already-present, relatively long-lived Oct4 protein via a direct interaction. This is supported by the observation that the Cdx2 mutant phenotype is far more severe than that of the AP2α/γ-deficient embryo. Furthermore, when Cdx2′s repressive function is counteracted by high levels of Oct4, as seen in cattle blastocyst TE, the presence of AP2 proteins is not sufficient to shut down Oct4 (*mOct4-GFP* into cattle experiment).

Importantly, neither Oct4-repression mechanism is active in cattle blastocyst TE with the resulting high Oct4 levels correlating with a delay in TE commitment. What is the evolutionary significance of this difference? The answer may lie in the different developmental pathways of mice and cattle. Mouse embryos implant a day after blastocyst formation and undergo a flurry of trophoblast proliferation and differentiation into giant cells, ectoplacental cone and extraembryonic ectoderm, before initiating gastrulation. Thus, there may be a need to shut off *Oct4* transcription rapidly, in particular as Oct4 protein is very stable. Cattle embryos float another 2 weeks in the uterus, during which time they undergo gastrulation (from E14) and proceed to neurula stages, before finally attaching to the endometrium. Intense TE proliferation only commences at E13, differentiation into binucleate cells (corresponding to mouse giant cells) at E15 (Berg et al., 2010). Hence, there are different demands on the TE lineages of these species. Cattle may simply not need to commit TE early and thus have no necessity to rapidly and actively shut down Oct4.

Mice and cattle also appear to use at least some distinct pathways in trophoblast specification (Smith et al., 2010). In particular, Eomes is a direct target of Cdx2 in mice and is required for trophoblast maintenance (Strumpf et al., 2005). While readily measurable in nascent mesoderm, neither we, nor others (Degrelle et al., 2005), have been able to detect Eomes during cattle trophoblast development, suggesting that this gene is not required for Cdx2 function in cattle. Interestingly, Eomes has been shown to be negatively regulated by Oct4 in mouse ES cells (Babaie et al., 2007; Loh et al., 2006), thus furnishing another reason why Oct4 may have to be controlled more tightly in the mouse TE.

In conclusion, we have uncovered interspecies diversity in the fixation of the first lineage, based on evolutionary divergence of Oct4 regulation, resulting in changes in Cdx2:Oct4 ratios and the relative timing of trophectoderm commitment. Considering that the absence of Oct4 restriction to the ICM is seen not only in cattle, but also in human, pig and rabbit blastocysts (Cauffman et al., 2005; Chen et al., 2009; Hall et al., 2009; Kirchhof et al., 2000; Kobolak et al., 2009; van Eijk et al., 1999), it appears that cattle may be more representative than mice of early mammalian development. Considering that the cause of the absence of Oct4 restriction,

namely, divergence in Oct4's enhancer elements, is also more general, differences in ES cell characteristics and responses in these species would be expected. Presumably such ES cells might be more permissive to AP2 (and potentially Cdx2) protein expression as Oct4 regulation is more refractile to these factors. Our work indicates the usefulness of performing functional studies in more than a single model system. This applies particularly when examining developmental events, such as placentation, that are relatively novel from an evolutionary standpoint.

EXPERIMENTAL PROCEDURES

Bovine Embryo Production

Cattle oocytes from local abbatoirs were aspirated, maturated and fertilized and zygotes cultured in vitro for 7 days in biphasic SOF medium (ESOF and LSOF) supplemented with 10 μmol 2,4-dinitrophenol from day 5 to 7 as described (Thompson et al., 2000). E7 embryos refer to selected late (expanded blastocoels) stage embryos collected 7 days postfertilization. Older embryos were obtained by transcervically transferring batches of Grade 1 and 2 blastocysts into the uterine horn of estrus-synchronized recipient cows (Berg et al., 2010). Embryos of the appropriate age were recovered by nonsurgical flushing (Berg et al., 2010). Animal procedures were conducted under the approval of the Ruakura Animal Ethics Committee (R.A.E.C. 11183).

Embryo Manipulations

Bovine ICMs were isolated from day 7 expanded blastocysts using immunosurgery (Solter and Knowles, 1975). Zonae pellucida were removed by a short incubation in 0.5% pronase (Sigma), then blastocysts were washed twice in HEPES buffered LSOF (Thompson et al., 2000) containing 0.5 mg/ml of polyvinyl alcohol (HSOF/PVA) and placed in rabbit anti-bovine Serum (Sigma 3759) for 40 min, washed twice in HSOF/PVA, and incubated for 30–40 min in guinea pig Complement Serum (Sigma 1639). The lysed trophectodermal cells were manually removed using a finely pulled pipette. The isolated ICM cells were washed twice in HSOF/PVA and vortexed in 100 μl TRIZOL (Sigma) for subsequent RNA isolation.

Trophectodermal cells were isolated from day 7 zona-intact expanded blastocysts by manually splitting the blastocyst into two portions, one containing the ICM and overlying polar trophectoderm, the other the mural trophectoderm. Lids of 35 mm dishes were coated with poly-L-lysine (Sigma P-8920) and allowed to dry overnight. Before use, lids were rinsed three times and then filled with HSOF. Blastocysts were placed onto lids and manually split into two pieces using an Ultra Sharp Splitting Blade (Bioniche, ESE020). The mural TE portion was aspirated from the zona with a finely pulled glass pipette, washed three times in HSOF/PVA, and vortexed in 100 μl TRIZOL for subsequent RNA isolation. If trophectodermal cells attached to the coated lids during or after splitting, a 20% w/v BSA solution was pipetted underneath the cells to allow intact detachment.

Expression Analyses

Whole-mount in situ hybridization was done as described (Donnison et al., 2005; Smith et al., 2007), but using 400 μl washes, 4-well plates (Nunc 176740), and moving embryos rather than exchanging solutions. E7 embryos were fixed 30 min at RT in 4% PFA/PBT. Permeabilization with 5 μg/ml proteinase K was 5 min for zona-intact and 30 s for zona-free embryos. For elevated temperatures, prewarmed solutions and heated stages were used. Plates were covered with thermofilm during overnight hybridizations.

Immunocytochemistry

After zona removal, blastocysts were fixed as above, washed in PBT, then 30 min in PBX (PBS/0.5% Triton X-100), blocked 1 hr in 5% lamb serum/PBX, rocked O/N, 4°C, with anti Cdx2 (Abcam, ab74339; 1:100) or anti Oct4 (Santa Cruz, sc-9081, 1:100 to 1:250), washed three times 1 hr, RT, in PBX, incubated O/N, 4°C, with donkey anti-rabbit Alexa Fluor 488 or 568 (1:500, Molecular Probes), washed three times with PBX and nuclei stained with Hoechst 33342.

RNA isolation, spike addition, reverse transcription, real-time PCR and quantitation procedures were performed as described (Smith et al., 2007) with modifications and primers detailed in Supplemental Experimental Procedures.

Expression of fluorescent proteins was monitored using Leica DMIRB and DMI6000B inverted microscopes with N2.1/GFP filter sets or an Olympus FV1000 confocal microscope.

Transgenic Embryo Production

Transgene construction is outlined in Supplemental Experimental Procedures.

Plasmid-free linearized DNA was injected at 2–3 ng/μl into FVB pronuclei to generate transgenic mouse embryos.

Transgenic cattle embryos were obtained by stably transfecting primary bovine fibroblast cells, then using these cells as donors for somatic cell nuclear transfer (SCNT) as described (Smith et al., 2007), though omitting the serum starvation step to prevent potential epigenetic shutdown of transgenes. For all constructs at least two different cell lines were used.

mOct4ΔPE-GFP (GOF-18 ΔPE GFP) embryos were derived from an established mouse line (Yoshimizu et al., 1999).

Cdx2 Knockdown

Bovine *Cdx2* knockdown oligos were designed using BLOCK-iT (Invitrogen) and inserted into Polymerase II-driven miR sequences downstream of EmGFP (Supplemental Experimental Procedures). Efficacy was tested by cotransfection of CHO cells with equimolar amounts of *Cdx2* (or control) knockdown vector and a target vector, in which the Cdx2-miR target site was inserted downstream of a red fluorescent reporter in the vector *pIRES2-DsRedExpress*. Control cells fluoresced in various shades of yellow depending on the relative amounts of DsRed and EmGFP expressed, whereas no red fluorescence could be detected when using the *Cdx2-miR712*-expressing construct (data not shown).

Electrophoretic Mobility Shift Assays

Oct4 CR4 probes were generated by PCR (primers in Supplemental Experimental Procedures), SalI restricted, and 0.1 pmol fill-in labeled O/N at 15°C using Klenow and 3 μl 3000 Ci/mmol ^{32}P-dCTP. column-purified labeled probe (1–2 fmol) was used per reaction. Whole cell extracts were prepared (Pfeffer et al., 2000) from mouse TS cells, TS cells grown for 5 days without FGF4/heparin or conditioned medium, mouse R1 ES cells (containing 0.1% MEFs) and feeder-free bovine trophectoderm line FT29.8 generously donated by Dr. Neil Talbot (Talbot et al., 2007). Extracts (0.1 μl) were used per reaction. Reaction (20 μl) conditions were 4% Ficoll, 1 mM EDTA, 10 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 20 ng/μl poly-dIdC, and 0.25 μg/μl BSA. Competitive oligos were added at 200-fold molar excess. AP2 Antibodies (0.2 μg; Santa Cruz; sc-12726 and sc-8977) were preincubated for 10 min before probe addition. Reactions were kept on ice for 30 min then electrophoresed on 6% polyacrylamide/0.25x TBE prerun gels.

Luciferase and ChIP Assays

R1 ES cells plated 24 hr previously on 0.1% gelatine coated 12-well plates (50000 cells/well) were lipofected (Lipofectamine, Invitrogen) in triplicate with 500 ng pGL3-mCR4-Oct4Prom-Luciferase reporter, 500 ng empty or protein-expressing vector, and 10 ng pRL-TK plasmid (Promega). We avoided the use of pRL-CMV to normalize expression levels as this construct was strongly repressed specifically by AP2 proteins. Twenty-four hours after transfection, luciferase activities were measured with the dual luciferase assay system (Promega). pGL3-mCR4-Oct4Prom-Luciferase reporter (kindly provided by Dr. F. Ishikawa) contains the mouse Oct4 distal enhancer CR4 region (−2185/−1946) (Figure 6A) linked to the endogenous *Oct4* promoter (−284 to the ATG) (Okumura-Nakanishi et al., 2005). The mouse Cdx2, Tcfap2a, Tcfap2c, Oct4, and Pax2 cDNAs were cloned into the expression vector pKW2T (kind gift from Dr. M. Busslinger) and expression verified by SDS-gel analysis after in vitro transcription/translation.

Two million mouse R1 ES or TS cells were used per 2 μg of AP2γ antibody (SantaCruz, H77) for chromatin immunoprecipitation (ChIP), following the protocol of Nelson and co-workers (Nelson et al., 2009). For real-time PCR quantitation, Oct4 CR4 primers CTGGGAGGAACTGGGTGTGGGGAGGTTGTA and CTTCCTCAATAGCAGATTAAGGAAGGGCTA and Elf5 intron 1 primers AAATCCTCAGGACGCTCAGCGG and TCTGACTTTCTTGCAGGCGTGC were used.

Lineage Tracing and Aggregation Experiments

Embryo Labeling

A stock solution (2 μg/ml) CellTracker CM-DiI dye (Molecular Probes, C-7000) was prepared in tissue-grade DMSO, aliquoted and stored at −20°C until use. Bovine TE cells were specifically labeled by washing expanded zona-free blastocysts three times in PBS/PVA, staining with 0.05 μg/ml of CM-DiI for 4 min at 37°C before extensive washing in PBS/PVA to remove excess stain. Pilot experiments with blastocysts labeled as above, cultured overnight, fixed and examined by confocal microscopy, confirmed that staining was restricted to the TE. For early gastrula embryos (E14), the epiblast was dissected away after labeling.

TE Dissociation

Labeled expanded bovine blastocysts were equilibrated for up to an hour under 5% CO_2 in LSOF culture drops before dissociating the cells. Blastocysts were washed three times in transfer SOF (TSOF = Ca^{2+}- and DNP-free HSOF), then incubated for 20 min at 38.5°C in TSOF containing 0.5 μg/ml Cytocholasin D (Sigma 8273). Blastocysts were washed three times in PBS/PVA then incubated for 10–15 min at 37°C in 0.25% Trypsin/5% EDTA in PBS/PVA. Trypsin was inactivated by incubating individual blastocysts in 20 μl drops containing PBS/10% FCS for 5 min. After transferral to 20 μl drops of 3 mg BSA/ml TSOF, a fine bore glass mouth pipette was used to dissociate embryos into individual cells or clumps of two to three cells. Only fluorescing cells, kept in TSOF/2% FCS, were used for aggregation.

Aggregation

Aggregation of TE cells and 8 cell embryos was done in microwells (Vajta et al., 2000) melted into 35 mm Petri dishes (Falcon 1007), one microwell per 10 μl drop of ESOF, overlaid with 3 ml of mineral oil (Sigma). A zona-free 8 cell (50 hr postinsemination) embryo was placed in the bottom of the microwell and six to eight CM-DiI labeled TE cells were pushed over the edge of the microwell and allowed to settle onto the embryo. Another 8 cell embryo was gently placed in the microwell over the top of the TE cells to form a "sandwich." Aggregates were in vitro cultured to blastocyst stages (96 hr additional culture after aggregation) with ESOF medium changed to LSOF 48 hr after aggregation. Development controls consisted of two 8 cell embryos without added TE cells. Embryos were washed in PBS/PVA, incubated in Hoechst 33342 for nuclear staining and fixed in 4% PFA/PBS for 30 min at RT. After confocal microscopy, cells were independently scored by two researchers in a blind assay for contribution to the ICM and TE. In six experimental sessions, 59 blastocyst and 74 E14 gastrula TE aggregates were performed. Development to blastocyst was 66% for blastocyst- and 55% for gastrula-derived TE aggregates and between 50% and 81% for control aggregates.

Transgenic Aggregations

Long-term development (to E16 gastrula-stages) was followed using chimeras consisting of host nuclear transfer-derived β-Gal expressing 8 cell embryos and E7 TE cells from expanded wild-type (in vitro produced) blastocysts. For the generation of the transgenic host embryos, bovine fibroblasts were stably transfected with a *pCAG-lacZ-IRES-puro* construct, generated as described in Supplementary Information. SCNT was performed as described above. Embryos were transferred to recipient cows at E7 and retrieved at E16, stained for β-Gal activity (Pfeffer et al., 2000), embedded in paraffin, and sectioned.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at doi:10.1016/j.devcel.2011.01.003.

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REFERENCES

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. *17*, 126–140.

Babaie, Y., Herwig, R., Greber, B., Brink, T.C., Wruck, W., Groth, D., Lehrach, H., Burdon, T., and Adjaye, J. (2007). Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells *25*, 500–510.

Balakier, H., and Pedersen, R.A. (1982). Allocation of cells to inner cell mass and trophectoderm lineages in preimplantation mouse embryos. Dev. Biol. *90*, 352–362.

Berg, D.K., van Leeuwen, J., Beaumont, S., Berg, M., and Pfeffer, P.L. (2010). Embryo loss in cattle between days 7 and 16 of pregnancy. Theriogenology *73*, 250–260.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947–956.

Cauffman, G., Van de Velde, H., Liebaers, I., and Van Steirteghem, A. (2005). Oct-4 mRNA and protein expression during human preimplantation development. Mol. Hum. Reprod. *11*, 173–181.

Chen, A.E., Egli, D., Niakan, K., Deng, J., Akutsu, H., Yamaki, M., Cowan, C., Fitz-Gerald, C., Zhang, K., Melton, D.A., et al. (2009). Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. Cell Stem Cell *4*, 103–106.

Chew, J.L., Loh, Y.H., Zhang, W., Chen, X., Tam, W.L., Yeap, L.S., Li, P., Ang, Y.S., Lim, B., Robson, P., et al. (2005). Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol. Cell. Biol. *25*, 6031–6046.

Degrelle, S.A., Campion, E., Cabau, C., Piumi, F., Reinaud, P., Richard, C., Renard, J.P., and Hue, I. (2005). Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. Dev. Biol. *288*, 448–460.

Dietrich, J.E., and Hiiragi, T. (2007). Stochastic patterning in the mouse preimplantation embryo. Development *134*, 4219–4231.

Donnison, M., Beaton, A., Davey, H.W., Broadhurst, R., L'Huillier, P., and Pfeffer, P.L. (2005). Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. Development *132*, 2299–2308.

Dyce, J., George, M., Goodall, H., and Fleming, T.P. (1987). Do trophectoderm and inner cell mass cells in the mouse blastocyst maintain discrete lineages? Development *100*, 685–698.

Fleming, T.P. (1987). A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. Dev. Biol. *119*, 520–531.

Georgiades, P., and Rossant, J. (2006). Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. Development *133*, 1059–1068.

Guo, G., Huss, M., Tong, G.Q., Wang, C., Li Sun, L., Clarke, N.D., and Robson, P. (2010). Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Dev. Cell *18*, 675–685.

Hall, V.J., Christensen, J., Gao, Y., Schmidt, M.H., and Hyttel, P. (2009). Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. Dev. Dyn. *238*, 2014–2024.

Handyside, A.H. (1978). Time of commitment of inside cells isolated from preimplantation mouse embryos. J. Embryol. Exp. Morphol. *45*, 37–53.

Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M., and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J. Biol. Chem. *284*, 28729–28737.

Keefer, C.L., Pant, D., Blomberg, L., and Talbot, N.C. (2007). Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. Anim. Reprod. Sci. *98*, 147–168.

Kidder, B.L., and Palmer, S. (2010). Examination of transcriptional networks reveals an important role for TCFAP2C, SMARCA4, and EOMES in trophoblast stem cell maintenance. Genome Res. *20*, 458–472.

Kimber, S.J., Sneddon, S.F., Bloor, D.J., El-Bareg, A.M., Hawkhead, J.A., Metcalfe, A.D., Houghton, F.D., Leese, H.J., Rutherford, A., Lieberman, B.A., et al. (2008). Expression of genes involved in early cell fate decisions in human embryos and their regulation by growth factors. Reproduction *135*, 635–647.

Kirchhof, N., Carnwath, J.W., Lemme, E., Anastassiadis, K., Scholer, H., and Niemann, H. (2000). Expression pattern of Oct-4 in preimplantation embryos of different species. Biol. Reprod. *63*, 1698–1705.

Kobolak, J., Kiss, K., Polgar, Z., Mamo, S., Rogel-Gaillard, C., Tancos, Z., Bock, I., Baji, A.G., Tar, K., Pirity, M.K., et al. (2009). Promoter analysis of the rabbit POU5F1 gene and its expression in preimplantation stage embryos. BMC Mol. Biol. *10*, 88.

Kuckenberg, P., Buhl, S., Woynecki, T., van Furden, B., Tolkunova, E., Seiffe, F., Moser, M., Tomilin, A., Winterhager, E., and Schorle, H. (2010). The transcription factor TCFAP2C/AP-2gamma cooperates with CDX2 to maintain trophectoderm formation. Mol. Cell. Biol. *30*, 3310–3320.

Kuijk, E.W., Du Puy, L., Van Tol, H.T., Oei, C.H., Haagsman, H.P., Colenbrander, B., and Roelen, B.A. (2008). Differences in early lineage segregation between mammals. Dev. Dyn. *237*, 918–927.

Kurosaka, S., Eckardt, S., and McLaughlin, K.J. (2004). Pluripotent lineage definition in bovine embryos by Oct4 transcript localization. Biol. Reprod. *71*, 1578–1582.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. *38*, 431–440.

Maddox-Hyttel, P., Alexopoulos, N.I., Vajta, G., Lewis, I., Rogers, P., Cann, L., Callesen, H., Tveden-Nyborg, P., and Trounson, A. (2003). Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. Reproduction *125*, 607–623.

McPherson, L.A., and Weigel, R.J. (1999). AP2alpha and AP2gamma: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. Nucleic Acids Res. *27*, 4040–4049.

Nelson, J., Denisenko, O., and Bomsztyk, K. (2009). The fast chromatin immunoprecipitation method. Methods Mol. Biol. *567*, 45–57.

Nishioka, N., Inoue, K., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R.O., Ogonuki, N., et al. (2009). The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev. Cell *16*, 398–410.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell *123*, 917–929.

Nordhoff, V., Hubner, K., Bauer, A., Orlova, I., Malapetsa, A., and Scholer, H.R. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. Mamm. Genome *12*, 309–317.

Okumura-Nakanishi, S., Saito, M., Niwa, H., and Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. J. Biol. Chem. *280*, 5307–5317.

Pfeffer, P.L., Bouchard, M., and Busslinger, M. (2000). Pax2 and homeodomain proteins cooperatively regulate a 435 bp enhancer of the mouse Pax5 gene at the midbrain-hindbrain boundary. Development *127*, 1017–1028.

Ralston, A., and Rossant, J. (2008). Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. Dev. Biol. *313*, 614–629.

Ralston, A., Cox, B.J., Nishioka, N., Sasaki, H., Chea, E., Rugg-Gunn, P., Guo, G., Robson, P., Draper, J.S., and Rossant, J. (2010). Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. Development *137*, 395–403.

Rossant, J., and Vijh, K.M. (1980). Ability of outside cells from preimplantation mouse embryos to form inner cell mass derivatives. Dev. Biol. *76*, 475–482.

Smith, C., Berg, D., Beaumont, S., Standley, N.T., Wells, D.N., and Pfeffer, P.L. (2007). Simultaneous Gene Quantitation of Multiple Genes in Individual Bovine Nuclear Transfer Blastocysts. Reproduction *133*, 231–242.

Smith, C.S., Berg, D.K., Berg, M., and Pfeffer, P.L. (2010). Nuclear transfer-specific defects are not apparent during the second week of embryogenesis in cattle. Cell. Reprogram. *12*, 699–707.

Solter, D., and Knowles, B.B. (1975). Immunosurgery of mouse blastocyst. Proc. Natl. Acad. Sci. USA *72*, 5099–5102.

Spindle, A.I. (1978). Trophoblast regeneration by inner cell masses isolated from cultured mouse embryos. J. Exp. Zool. *203*, 483–489.

Stern, M.S., and Wilson, I.B. (1972). Experimental studies on the organization of the preimplantation mouse embryo. I. Fusion of asynchronously cleaving eggs. J. Embryol. Exp. Morphol. *28*, 247–254.

Strumpf, D., Mao, C.A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F., and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development *132*, 2093–2102.

Suwinska, A., Czolowska, R., Ozdzenski, W., and Tarkowski, A.K. (2008). Blastomeres of the mouse embryo lose totipotency after the fifth cleavage division: expression of Cdx2 and Oct4 and developmental potential of inner and outer blastomeres of 16- and 32-cell embryos. Dev. Biol. *322*, 133–144.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Talbot, N.C., Powell, A.M., Camp, M., and Ealy, A.D. (2007). Establishment of a bovine blastocyst-derived cell line collection for the comparative analysis of embryos created in vivo and by in vitro fertilization, somatic cell nuclear transfer, or parthenogenetic activation. In Vitro Cell. Dev. Biol. Anim. *43*, 59–71.

Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072–2075.

Thompson, J.G., McNaughton, C., Gasparrini, B., McGowan, L.T., and Tervit, H.R. (2000). Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J. Reprod. Fertil. *118*, 47–55.

Vajta, G., Peura, T.T., Holm, P., Paldi, A., Greve, T., Trounson, A.O., and Callesen, H. (2000). New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. Mol. Reprod. Dev. *55*, 256–264.

van Eijk, M.J., van Rooijen, M.A., Modina, S., Scesi, L., Folkers, G., van Tol, H.T., Bevers, M.M., Fisher, S.R., Lewin, H.A., Rakacolli, D., et al. (1999). Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. Biol. Reprod. *60*, 1093–1103.

Van Soom, A., Boerjan, M., Ysebaert, M.T., and De Kruif, A. (1996). Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. Mol. Reprod. Dev. *45*, 171–182.

Van Soom, A., Boerjan, M.L., Bols, P.E., Vanroose, G., Lein, A., Coryn, M., and de Kruif, A. (1997). Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. Biol. Reprod. *57*, 1041–1049.

Vejlsted, M., Avery, B., Schmidt, M., Greve, T., Alexopoulos, N., and Maddox-Hyttel, P. (2005). Ultrastructural and immunohistochemical characterization of the bovine epiblast. Biol. Reprod. *72*, 678–686.

Winger, Q., Huang, J., Auman, H.J., Lewandoski, M., and Williams, T. (2006). Analysis of transcription factor AP-2 expression and function during mouse preimplantation development. Biol. Reprod. *75*, 324–333.

Yagi, R., Kohn, M.J., Karavanova, I., Kaneko, K.J., Vullhorst, D., DePamphilis, M.L., and Buonanno, A. (2007). Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development *134*, 3827–3836.

Yeom, Y.I., Fuhrmann, G., Ovitt, C.E., Brehm, A., Ohbo, K., Gross, M., Hubner, K., and Scholer, H.R. (1996). Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development *122*, 881–894.

Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y.I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Scholer, H.R., and Matsui, Y. (1999). Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev. Growth Differ. *41*, 675–684.